CCLXXXII. THE NATURE OF THE FATTY ACIDS STORED BY THE LIVER IN THE FAT-DEFICIENCY DISEASE OF RATS

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THE part played by those unsaturated fatty acids which have been shown to be essential for the maintenance of rats in normal health is as yet quite unknown. A first step in elucidating this problem was to gain further knowledge as to the nature of the fatty acids stored when the animals were kept on a fat-deficient diet, and to observe the changes which occurred in them when the curative acids were fed.

At the conclusion of feeding experiments carried out in co-operation with Hume *et al.* [1938], we selected five groups of rats and examined the fatty acids present in the livers. The long period of preparation necessary before symptoms are sufficiently well established for curative measures to be tested made strict economy desirable and in most cases before the rats were killed the negative controls had been utilized for further tests. It was not, therefore, possible to obtain a large group of negative controls for the isolation of the liver fatty acids and we had not sufficient material to make a separation into phospholipin and neutral fat. In normal animals the greater part of the liver fatty acids comes from the phospholipins; in the ox, Bloor & Snider [1930] give the proportion of phospholipin fatty acid to acid from neutral fat as 6 : 1; this figure is in close agreement with those given by Klenk & Schoenebeck [1932]. Theis [1928], on the other hand, gives the ratio as 5 : 4. Monaghan [1932] found that in fasting animals the proportion of neutral fat was much raised. It seems probable that most of the highly unsaturated fatty acids came from the phospholipin fraction, but definite evidence on this point is still required.

The five groups of rats selected were:

(1) *Two rats* (♂+♀) *which served as our negative controls.* These weighed 42 and 45 g. respectively when placed on the fat-free diet. The diet was continued for 18 weeks and after the growth curve had remained flat for 6 weeks they received respectively daily doses of 0·2 g. linusic and *iso*linusic acids. These doses were continued for 5 weeks, during which period the weight of the linusic-fed rat increased by 5 g. and that of the one fed with *iso*linusic by 8 g. For the next 7½ weeks before being killed they again received the fat-deficient diet; these were chosen as being the nearest to completely negative controls available at the end of the feeding experiments.

Although the linusic-fed rat only gained 5 g. in weight during the 5 weeks period of dosing, 17½ g. increase was recorded in the 3½ weeks immediately following cessation of the dose, then the weight remained stationary until the rat was killed 3½ weeks later. Since both rats had received the fat-free diet for 7 weeks before they were killed and prior to that the comparatively inactive hexahydroxy-acids had been fed, these rats may probably be regarded as characteristic examples of rats fed on a fat-free diet.

(2) *Rats receiving methyl linoleate.* Three rats (♀) weighing 44, 45 and 46 g. respectively when transferred to the fat-deficient diet, were utilized. One of these, after it had been on this diet for 20 weeks, was given a daily dose of 6 drops methyl linoleate for a period of 6 weeks during which its weight rose from 121 to 152 g. A month's cessation of dosing followed during which the rat's weight increased by 16 g. and finally, for its last 3 weeks, it received 1 drop linoleate daily. The other two rats, after receiving the fat-deficient diet for 6 months, were both dosed with 1 drop linoleate daily for 38 days, the respective gains in weight being 12 and 15 g. This dose was shown to produce amelioration but not cure of symptoms and the rats, therefore, probably do not show the typical results of rats fed with the optimum linoleate dose.

(3) *Rats receiving methyl linolenate.* Two rats (♀) which had received the fat-free diet for 16 weeks were given, respectively, during the course of 1 month, a daily dose of 0·2 g. dioxidostearic acid and 1 drop of methyl linolenate. Finally for 38 days before they were killed, each received 6 drops linolenate daily. The effects produced may therefore be considered as characteristic for rats fed with methyl linolenate.

(4) *Rats receiving linseed oil.* Six rats (3 ♂, 3 ♀) had received the fat-free diet for from 8 to 10 months. After 4 months one had been given 0·2 g. linusic acid daily for 5 weeks and then after a month's interval a daily dose of 0·2 g. trihydroxystearic acid for another period of 5 weeks. In all cases for 2 to 3 months before being killed, each rat had received a supplement of 5 to 15 drops linseed oil daily. The typical fatty acids stored by rats on a diet containing linseed oil might, therefore, be expected.

(5) *Rats fed with docosahexaenoic acid methyl ester (from cod liver oil).* Two rats were utilized (1 ♂, 1 ♀) which had served as negative controls for 6 months. During 2 weeks they each received a daily dose of 8 drops of the above ester [Farmer & Van den Heuvel, 1938] and for the subsequent 3 weeks, 5 drops daily. Dosage had ceased a fortnight before the rats were killed. Possibly if the rats had been killed whilst the diet still contained the ester supplement, the I.V. of the liver fatty acids might have been higher.

As soon as the animals were killed, the livers were removed, weighed and immediately thrown into a mixture of equal parts of alcohol, water and caustic potash heated on a water bath. After it had been extracted with ether, the soap solution was acidified and the fatty acids were extracted by light petroleum.

Table I

Supplement	No. rats	Total wt. livers g.	Av. wt. liver per rat g.	Total wt. fatty acids g.	Wt. acids in 100 g. liver g.	% acids (a) unsaturated	% acids (b) saturated	I.V. unsaturated acids
Group I. None	2 (♂+♀)	16·5	8·2	0·62	3·7	62·1	37·9	121·2
Group II. Methyl linoleate	3 (♀)	21·8	7·3	0·57	2·6	62·1	37·9	163·6
Group III. Methyl linolenate	2 (♀)	15·0	7·5	0·36	2·4	62·3	37·7	215·0
Group IV. Linseed oil	6 (3♂+3♀)	55·0	9·2	1·25	2·3	61·7	38·3	202·8
Group V. Methyl ester of cod liver oil acid (docosahexaenoic acid)	2 (♂+♀)	17·3	8·7	0·50	2·9	61·6	38·4	162·3

Three interesting facts emerge from the results recorded in Table I:

(1) The weight of fatty acid calculated per 100 g. liver tissue was considerably higher in the negative controls than in the rats which had received a supplement of unsaturated acid.

(2) Whatever the diet, the proportion of the weights of saturated and unsaturated acids remained extraordinarily constant, the ratio being 38 : 62. Sinclair [1935] found that the proportion of saturated to unsaturated acids in the rat liver phospholipin fraction was 33·6 : 60·9.

(3) The iodine value of the unsaturated acids varied greatly, that of the acids from the negative controls being much lower than that from those rats which had received the doses of the esters of the unsaturated acids.

The rats which had been fed with the linolenic ester or with linseed oil contained the most highly unsaturated fats in their livers. This may possibly be explained by the fact that the dose of linoleate fed to Group II of the rats was known to be well below the optimum and that in the case of the rats dosed with the cod liver oil acid, the dose had ceased a fortnight before the rats were killed.

After the iodine value had been determined, each unsaturated fraction was brominated in ether solution at 0°; after standing overnight in the cold room, the solid ether-insoluble bromides were separated, washed well with ether, extracted with benzene, dried to constant weight and the bromine was estimated. The original weight of unsaturated acid present in the ether-insoluble bromides was then calculated. After the ether-soluble bromides had been weighed they were divided into a liquid fraction soluble in light petroleum and a solid residue small in amount and insoluble in light petroleum. The percentage of bromine was then determined in the petroleum-soluble fraction and the corresponding weight of unsaturated acid calculated. These results are set forth in Table II.

Table II

Supplement to fat-deficient diet	Wt. acids brominated g.	Ether-insoluble bromides			Ether-soluble bromides: Total wt. g.	(a) Petroleum-insoluble			(b) Petroleum-soluble liquids		% of total acids (calculated from % Br in bromides)		
		Wt. g.	M.P. °C.	% Br.		Wt. g.	M.P. °C.	% Br	Wt. g.	% Br	Ether insol.	Petroleum Insol.	Petroleum Sol.	Total
Group I:		(a) Benzene-soluble												
None	0·2576	0·0264	203–4	61·8	0·41	0·04	190–5	—	0·368	39·76	3·8	5·9[1]	86·0	95·7
Group II:		(b) Benzene-insoluble												
Methyl linoleate	0·2459	0·0376	226–30[2]	64·3	0·43	0·07	200–5	—	0·363	44·78	5·3	10·9[1]	84·3	100·5
Group III:														
Methyl linolenate	0·1406	0·0831	Decomp.[3] above 260	69·0	0·26	0·11	145–8 with decomp.	—	0·149	45·94	17·6	28·7[2]	57·0	103·3
Group IV:														
Linseed oil	0·6506	0·2287	Decomp.[3] above 260	—	1·13	0·27	140–5 with decomp.	—	0·859	42·96	—	15·2[2]	57·0	—
Group V:														
Methyl ester of cod liver oil acid	0·2450	0·0908	Decomp.[3] above 260	70·76	0·41	0·14	—	62·3	0·263	39·1	16·1	21·6	67·0	104·7

[1] Calculated on the assumption that the bromides contain 61·8% Br.
[2] Calculated on the assumption that the bromides contain 63·3% Br.
[3] Traces of bromide of similar M.P. were found in the benzene extract.
Theory for decabromodocosanic acid: 70·8% Br.
Theory for octabromodocosanic acid: 65·8% Br.
Theory for octabromoarachidonic acid: 67·7% Br.
Theory for hexabromoarachidonic acid: 61·06% Br.
Theory for hexabromostearic acid: 63·31% Br.

The nature of the unsaturated acids present in the liver

(1) *Group of negative controls.* The solid ether-insoluble bromide prepared from the negative controls was completely soluble in hot benzene and octa- or deca-bromides of acids containing 20 or 22 carbon atoms were therefore absent. The benzene-soluble bromide melting sharply at 203–4° does not seem to have been previously described. Linolenic hexabromide (M.P. 182–4°) was not present.

Analysis agreed with the formula $C_{20}H_{34}O_2Br_6$. (Found: Br, 61·80; C, 29·82; H, 3·97 %. $C_{20}H_{34}O_2Br_6$ requires Br, 61·06; C, 30·54; H, 4·32 %.) The bromide must, therefore, be derived from a C_{20} acid containing three ethylene linkages, possibly a dihydroarachidonic acid, but arachidonic acid itself is absent.

Sinclair [1935] separated the phospholipins from the total fat contained in the carcasses of rats fed on a fat-deficient diet and found that the ether-insoluble bromides contained 62·2 % Br, a number appreciably lower than that required for linolenic hexabromide (63·32 % Br); he suggested that unsaturated acids hitherto unidentified might be present in these controls. Since Sinclair's results were obtained on the phospholipin fraction, it is probable that the new hexabromide may also have been derived from phospholipin acid. The solubility of Sinclair's bromide in benzene was not definitely stated, nor did he not record its M.P., so that it is not possible to compare further these two substances. In our experiments the acid in the benzene-soluble bromide formed only 3·8 % of the total acids. A further 5·9 % of what appeared to be a less pure specimen of the same acid separated from the petroleum-insoluble fraction of the ether-soluble bromides. After recrystallization this melted at 190–195° but there was insufficient for analysis. In all, therefore, probably rather more than 9 % of the total fatty acid consisted of the dihydroarachidonic acid. The ether-soluble fraction contained bromide corresponding with 86 % of the total acids. The Br content was 39·76 %, so that in addition to oleic dibromide (36·19 % Br) some small proportion of liquid bromides of the more unsaturated acids must have been present.

There was no indication of the presence of either linoleic or linolenic acid.

(2) *Group fed with methyl linoleate.* Here the liver fatty acids yielded ether-insoluble bromides containing 64·3 % Br corresponding to a yield of 5·3 % higher unsaturated fatty acids. The bromide was insoluble in benzene; M.P. 226–30° with decomposition. From the ether-soluble bromide fraction a solid residue was obtained insoluble in light petroleum; when recrystallized, it melted at 200–205° and was probably therefore the dihydroarachidonic acid hexabromide isolated from the negative control; there was insufficient for analysis. The bromine percentage (64·3) in the benzene-insoluble bromide was less than that required by arachidonic octabromide (67·7). Since this fraction melted with decomposition at 226° it was possibly a mixture of arachidonic octabromide and dihydroarachidonic hexabromide, though if present the latter might have been expected to have been dissolved by the benzene. A somewhat similar fraction was obtained by Klenk & Schoenebeck [1932] in separating the acids of the phospholipins of ox liver. This melted with decomposition at 230–33° and by debromination and subsequent dehydrogenation was shown to consist of a mixture of C_{18} and C_{20} derivatives. It seems likely that a mixture of arachidonic acid and its dihydro derivative would behave similarly. Klenk and Schoenebeck identified considerable quantities of linoleic acid as the tetrabromide (M.P. 114–15°) but no evidence of this was found in the fat we examined. The separation of a bromide fraction insoluble in benzene and containing a higher % of Br than that in the C_{20} hexabromide indicate the presence of an acid containing four or more double bonds. As has already been pointed out, only minimal doses of linoleate had been fed to the rats in this group

and it is desirable that the fat should be investigated when adequate supplements of this acid were added to the fat-free diet.

The proportion of unsaturated acid derived from the ether-soluble bromide was 84·3 % of the total amount, in this respect closely resembling the state of affairs existing in the fat of the negative controls. The bromine content of the ether-soluble bromide was, however, higher (44·78 %), resembling that in the linolenic-fed rats.

(3) *Group of rats fed with methyl linolenate.* Here the amount of bromide insoluble in both ether and benzene corresponded to 17·6 % of the total acids. The bromide decomposed above 260° and contained 69·0 % Br; this would be in agreement with a mixture of arachidonic octabromide (67·7 % Br) and the deca-bromide of the C_{22} acid (70·8 % Br), or possibly a mixture of the octa- and deca-bromides of the C_{22} acid was present. The ether-soluble bromide fraction contained 45·94 % Br and corresponded to a mixture of oleic dibromide with liquid bromides of more unsaturated acids, the corresponding acids forming 57 % of the total. The petroleum-insoluble fraction of the ether-soluble bromides gave an indefinite M.P. at 145–8°; the bromide was not estimated but the corresponding acids probably formed more than 20 % of the whole amount present. Since linolenic hexabromide melts at 182–4°, the presence of an impure specimen of this is not excluded. Great caution has to be exercised in drawing deductions as to the nature of a bromide from its solubility when a large proportion of liquid bromides is also present in the solution.

(4) *Group fed with linseed oil.* Like that from the linolenic-fed rats, the benzene-insoluble bromide decomposed above 260° but unfortunately this fraction was accidentally lost before its weight and its Br content had been determined. The ether-soluble fraction contained 42·96 % Br and the corresponding acid formed 57 % of the total. There was also a fraction insoluble in light petroleum melting at 140–5°, the corresponding acid forming from 10 to 20 % of the whole. These results follow closely those obtained from the rats fed with linolenic acid.

(5) *Group fed with the methyl ester of the cod liver oil acid.* The benzene-insoluble bromide decomposed above 260°; analysis agreed with the formula for the bromide of docosapentaenoic acid $C_{22}H_{34}O_2Br_{10}$. (Found: C, 22·57; H, 3·01; Br, 70·8 %. Theory: C, 23·36; H, 2·72; Br, 70·8 %.) The free acid represented 16 % of the total fatty acids. The ether-soluble fraction also consisted of oleic dibromide with a small proportion of the bromides of the higher unsaturated acids, the latter forming 67 % of the total acids. The petroleum-insoluble fraction was a sticky solid containing 62·30 % Br; the debrominated acid therefore formed 21·6 % of the total.

Discussion

The absence of any acid containing 20 or 22 carbon atoms and four or more ethylene linkages in the rats fed on the completely fat-free diet suggests that unless linoleic or linolenic acid is given, the rat-liver is unable to synthesize the C_{20} or C_{22} acid containing four or more double bonds, essential for the continued existence of the animal. The source of the dihydroarachidonic acid isolated from the livers of the negative controls is not certain. It has been established, largely owing to the work of Sinclair, that the replacement of these highly unsaturated acids takes place extremely slowly, and this is corroborated by the long time that is necessary to establish the symptoms of the fat deficiency disease in rats which had been placed on the fat-free diet immediately after thay had been weaned. The dihydroarachidonic acid was, therefore, probably derived from arachidonic

acid originally present in the rat, its synthesis from the oleic or saturated acids which the rat may still be able to form from carbohydrate is not excluded.

It was unfortunate that the only linoleate-fed rats available were those which had been kept for a very long time on the fat-free diet and then fed for 5 weeks with quite inadequate amounts of linoleate, so that they could not be expected to show typical results. Small as were the doses of linoleate, they resulted in the deposition in the liver of an acid containing more than three double bonds. From the bromine content of this bromide fraction, a mixture of arachidonic and dihydroarachidonic acids may have been present. Possibly because of the insufficiency of the doses of linoleate which had been given, the C_{20} hexabromide, which had been isolated from the negative controls, appeared to be present in the petroleum-insoluble fraction; presumably its bromide had been carried into solution by the large proportion of ether-soluble liquid bromides present.

There is a good deal of evidence in the literature that arachidonic acid is formed when oils containing linoleic acid are fed [Ellis & Isbell, 1926; Eckstein, 1929; Spadola & Ellis, 1936]. There is not, however, always a clear differentiation made between the octabromide of arachidonic acid and the decabromide of the C_{22} acid.

Again from the literature [Ellis & Isbell, 1926; Klenk, 1932; Snider & Bloor, 1933] convincing evidence is present showing that linolenic acid is not stored as such in the body. Our experiments seem to point to its conversion into the C_{22} acid with five double bonds. The function of the liver here is not to desaturate the 18-carbon chain but, given the suitable condition of desaturation, to build up a longer chain, additional double bonds being formed in the process. The classification of linoleic acid as a vitamin appears to us to be undesirable in view of the fact that linoleic and linolenic acids seem to be the necessary building stones for the synthesis of arachidonic and docosapentaenoic acids. The latter acid was also obtained from the livers of rats fed with the docosahexaenoic acid isolated from cod liver oil, so that in this the hydrogenation of one double bond must have occurred. This hexaenoic acid was shown [Hume *et al.* 1938] to produce no amelioration of skin symptoms; it is as yet unkown whether these are cured by arachidonic acid, since Turpeinen [1938], who carried out experiments on rats in which this acid was fed, gives only a general statement about the healing of skin symptoms.

It seems that whereas the essential linoleic or linolenic acids may have some direct effect on the skin, their main function is to supply material from which higher and more unsaturated acids may be synthesized. Sinclair [1935] has stressed the care with which these are guarded and the length of time that elapses before they are lost from the phospholipins which contain them, and has concluded that these are not, like other lipins, concerned in the transport of fat but play some special role in the animal metabolism. It is, however, of interest that in our negative controls where these acids were absent, the percentage of fatty acid in the livers was higher than in the livers where these more highly unsaturated acids were present. In the fat-deficiency disease there is an inability to store fat, but there is some evidence that fat may still be synthesized from carbohydrate; it seems possible that the highly unsaturated acids may play some part in the storage of fat, either by enabling the more saturated fats to be carried to the depots or by influencing its passage into the connective tissue cells in which it is stored. The study of the basal metabolism and respiratory quotients made by Wesson and Burr [Wesson, 1927; Wesson & Burr, 1931] lends some support to this view.

SUMMARY

The livers of rats fed on a fat-free diet were free from acids containing four or more double bonds; a hitherto unknown acid $C_{20}H_{34}O_2$ was isolated as its hexabromide, melting at 202–4°. Rats which had been kept for a long time on the fat-free diet and then fed with very small doses of methyl linoleate probably contained the same dihydroarachidonic acid as had been found in the negative controls. Arachidonic or some more highly unsaturated acid was also present and must, therefore, have been synthesized from the linoleic acid given.

Rats fed on a fat-free diet and then dosed with methyl linolenate (6 drops daily) synthesized an acid which, from the bromine content of its bromide, appears to have been a mixture of arachidonic and docosapentaenoic acids. The C_{20} trienoic acid was not detected. A fortnight after dosing with the C_{22} hexaenoic acid (prepared from cod liver oil) had ceased, the liver contained the C_{22} pentaenoic acid. Linoleic and linolenic acids appear to be the building stones essential for the production of more highly unsaturated acids which play some unknown part in enabling the animal to store fat in its depots and tissues.

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